Ophiostoma gemellus and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa

Francois Roets[1]

Department of Botany and Zoology, Stellenbosch University, Stellenbosch, 7600, South Africa, and Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa

Z. Wilhelm de Beer
Michael J. Wingfield

Forestry and Agricultural Biotechnology Institute (FABI), Department of Microbiology, University of Pretoria, South Africa

Pedro W. Crous

Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, The Netherlands

Léanne L. Dreyer

Department of Botany and Zoology, Stellenbosch University, South Africa

Abstract: *Ophiostoma* (Ophiostomatales) represents a large genus of fungi mainly known from associations with bark beetles (Curculionidae: Scolytinae) infesting conifers in the northern hemisphere. Few southern hemisphere native species are known, and the five species that consistently occur in the infructescences of *Protea* spp. in South Africa are ecologically unusual. Little is known about the vectors of *Ophiostoma* spp. from *Protea* infructescences, however recent studies have considered the possible role of insects and mites in the distribution of these exceptional fungi. In this study we describe a new species of *Ophiostoma* and a new *Sporothrix* spp. with affinities to *Ophiostoma*, both initially isolated from mites associated with *Protea* spp. They are described as *Ophiostoma gemellus* sp. nov. and *Sporothrix variecibatus* sp. nov. based on their morphology and comparisons of DNA sequence data of the 28S ribosomal, β-tubulin and internal transcribed spacer (ITS1, 5.8S, ITS2) regions. DNA sequences of *S. variecibatus* were identical to those of a *Sporothrix* isolate obtained from *Eucalyptus* leaf litter in the same area in which *S. variecibatus* occurs in *Protea* infructescences. Results of this study add evidence to the view that mites are the vectors of *Ophiostoma* spp. that colonize *Protea* infructescences. They also show that DNA sequence comparisons are likely to reveal additional cryptic species of *Ophiostoma* in this unusual niche.

Key words: β-tubulin, ITS, LSU, phylogeny, vector

INTRODUCTION

Ophiostoma sensu lato Syd. & P. Syd. is a species-rich (ca. 140 species) ascomycete genus that includes many ecologically important taxa (Upadhyay 1981, Whitney 1982, Sinclair and Lyon 2005). Recent DNA-based phylogenetic reconstructions have identified three well supported monophyletic lineages in *Ophiostoma* that are tightly linked to morphological features such as the anamorph state or ascospore morphology (Zipfel et al 2006). Thus species with *Leptographium* Lagerb. & Melin anamorphs have been accommodated in the reinstated teleomorph genus *Grosmannia* Goid. Likewise *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. has been reinstated for species with short ascomatal necks, falcate ascospores, *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr. anamorphs and are sensitive to the antibiotic cycloheximide. Species with *Sporothrix* Hekt. & C.F. Perkins anamorphs and/or synnematous *Pesotum* J.L. Crane & Schokn. anamorphs have been retained in *Ophiostoma* Syd. & P. Syd.. The *Ophiostoma* group has substantial substructure linked to morphological characters and most likely will resolve into a number of distinct monophyletic lineages with the addition of more species and DNA loci (Zipfel et al 2006). It is treated as *Ophiostoma sensu stricto* in the present study.

Species of *Ophiostoma s. str.* typically produce ascospores in short-lived asci within flask-shaped ascomata. The ascospores are borne in gloeoid droplets at the tips of extended ascomatal necks. These characters represent adaptations to arthropod-mediated dispersal of their reproductive propagules (Münch 1907, 1908; Francke-Grosmann 1967; Upadhyay 1981; Malloch and Blackwell 1993). The vectors of *Ophiostoma* spp. include diverse arthropod taxa, such as bark beetles (Curculionidae: Scolytinae), longhorn beetles (Cerambycidae) and mites (Acari) (Barras and Perry 1975; Upadhyay 1981; Bridges and Moser 1983, 1986; Moser 1997, Jacobs and Wingfield 2001). The fungi are associated usually with galleries constructed in the phloem and wood of mainly coniferous trees by the larvae of the vector beetles

Accepted for publication 23 January 2008.

[1] Corresponding author. E-mail: fr@sun.ac.za

(Kirisits 2004). Apparent co-evolution between the fungi and their vectors has resulted in close associations that, at least in some instances, have been shown to be mutualistic (Francke-Grosmann 1967; Beaver 1989; Berryman 1989; Six and Paine 1998; Ayres et al 2000; Jacobs and Wingfield 2001; Klepzig et al 2001a, b).

Most species of *Ophiostoma* are known from the northern hemisphere. Where species have been recorded from southern hemisphere substrates, they are associated commonly with introduced insects or their origin is unknown (de Beer et al 1995, 1999; Zhou et al 2004, 2006). One of the most unusual and intriguing assemblages of seemingly native *Ophiostoma* spp. occurs in the infructescences (fruiting structures) of the endemic southern African genus *Protea* L. (*Proteaceae*), whose center of diversity is in the Cape Floristic region (Marais and Wingfield 1994, Rebelo 1995, Linder 2003). Five species of *Ophiostoma* have been described from *Protea* spp. in South Africa (Marais and Wingfield 1994, 1997, 2001; Roets et al 2006). Of interest, these fungi usually form the dominant fungal component within this protected environment (Roets et al 2005). The *Ophiostoma* spp. associated with *Protea* are not pathogenic to their hosts and have an ecological function that has yet to be defined (Roets et al 2005, 2006).

Ophiostoma africanum G.J. Marais & M.J. Wingf., *O. protearum* G.J. Marais & M.J. Wingf. and *O. splendens* G.J. Marais & M.J. Wingf., and the recently described *O. palmiculminatum* F. Roets et al and *O. phasma* F. Roets et al, have been isolated only from members of the culturally, ecologically and economically important host genus *Protea*, a genus that accommodates the national flower of South Africa (*P. cynaroides* L.). Phylogenetic analyses of DNA sequences for *Ophiostoma* spp. from *Protea* have revealed that these fungi are polyphyletic (Roets et al 2006). This suggests that there have been multiple invasions of this specialized niche.

Little is known about the vectors of the *Protea*-associated *Ophiostoma* spp. Their morphology however does suggest that insects or other small animals carry their spores between infructescences. In a preliminary attempt to find the vectors of the *Protea*-associated *Ophiostoma* spp., Roets et al (2007) identified the mites *Proctolaelaps vandenbergi* Ryke, two species of *Tarsonemus* Canestrini & Fonzago. and a single *Trichouropoda* Berlese sp. as the primary vectors of *O. palmiculminatum*, *O. phasma* and *O. splendens*. In that study they also isolated two unidentified species of *Sporothrix* from mites, one from a *Trichouropoda* sp. and the other from a *Tarsonemus* sp. Based on DNA sequence comparisons (Zipfel et al 2006, Roets et al 2007), both of these unidentified anamorph taxa also could be assigned to the teleomorph genus *Ophiostoma*. One of these species later produced teleomorph structures in culture. The aim of the present study was to identify the unknown *Ophiostoma* sp. and *Sporothrix* sp. based on morphological and physiological features, as well as comparisons of DNA sequences of the 28S ribosomal, β-tubulin and 5.8S rDNA (including the internal transcribed spacers 1 and 2) gene regions.

MATERIALS AND METHODS

Isolates.—Cultures used in this study included three isolates of the unknown *Ophiostoma* sp. (hereafter *O. gemellus*) and one isolate of the unknown *Sporothrix* sp. (hereafter *S. variecibatus*) collected from the surface of mites colonizing *Ophiostoma* containing *Protea* infructescences by Roets et al (2007) (TABLE I). These mites were stored at −20 C before they were crushed, vortexed in 2 mL ddH_2O, and plated on 2% malt-extract agar (MEA, Biolab, Midrand, South Africa) Petri dishes containing 0.04 g/L streptomycin sulphate and 0.05 g/L cycloheximide, which is selective for *Ophiostoma* spp. Additional isolates of both fungi were collected from *P. caffra* Meisn. (*O. gemellus*) and *P. longifolia* Andrews (*S. variecibatus*) (TABLE I). An isolate (CMW 2543) from *Eucalyptus* L'Her. leaf litter also was included because it was similar morphologically to *S. variecibatus* collected from *P. longifolia* as well as from mites. This isolate had been shown to be related to, but distinct from, *O. stenoceras* (Robak) Melin & Nannf. (de Beer et al 2003).

For morphological and physiological comparisons, representative isolates including the ex-type culture of *O. palmiculminatum* were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (TABLE I). All isolates were maintained in Petri dishes containing 2% malt-extract agar (MEA, Biolab, Midrand, South Africa) at 4 C. Representative cultures of the new taxa treated in this study have been deposited in both the CBS and the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Herbarium specimens of the anamorph and teleomorph structures of *O. gemellus* and anamorph structures of *S. variecibatus* were deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM) (TABLE I). DNA sequence data used for phylogenetic reconstructions of all other *Ophiostoma* spp. and isolates included in this study were obtained from GenBank (TABLE I).

Morphology and growth in culture.—Isolates of *O. gemellus* and *S. variecibatus* were grown in the dark for 8 d at 25 C on MEA (Biolab, Midrand, South Africa). Ascomata and conidiogenous cells that formed in culture were mounted on microscope slides in lactophenol (Stephens 1974). Specimens were studied with a Nikon Eclipse E600 light microscope with differential interference contrast. Photographic images were captured with a Nikon DXM1200 digital camera. Measurements (25) of each taxonomically

TABLE I. GenBank accession numbers for LSU, ITS and ß-tubulin nucleotide data for fungal isolates used in phylogenetic analysis

Species identity	Isolate no.		Host	Geographical origin	Collector	GenBank accession no.		
	CBS	CMW				LSU	ITS	B-tubulin
Ophiostoma sp. (Unknown) [=*O. gemellus* sp. nov.]	121957	23054	*Protea caffra*	W. Sisulu Park, South Africa	F. Roets		DQ821557	DQ821551
	21958	23056	*Protea caffra*	W. Sisulu Park, South Africa	F. Roets		DQ821558	DQ821552
		23055	*Protea caffra*	W. Sisulu Park, South Africa	F. Roets		DQ821559	DQ821553
	121959	23057	*Tarsonemus* sp. from *Protea caffra*	W. Sisulu Park, South Africa	F. Roets	DQ821531	DQ821560	DQ821554
		23058	*Tarsonemus* sp. from *Protea caffra*	W. Sisulu Park, South Africa	F. Roets	DQ821532	DQ821561	DQ821555
		23059	*Tarsonemus* sp. from *Protea caffra*	W. Sisulu Park, South Africa	F. Roets	DQ821533	DQ821562	DQ821556
O. abietinum		1468	*Dendroctonus ponderosa*	Canada	Y. Hiratsuka		AF484457	AY280468
		110	*Pinus echinata*	USA	F. Hinds		AF280488	AY280470
		109	*Pinus echinata*	USA	F. Hinds		AF280487	AY280469
O. africanum	116566	1104	*Protea caffra*	Irene, South Africa	Unknown	DQ316147	DQ316200	DQ316162
	116571	823	*Protea gaguedi*	Unknown	M.J. Wingfield	AF221015		
	116374	1822	*Protea dracomontana*	KZ-Natal, South Africa	M.J. Wingfield		DQ316179	DQ316159
O. aurorae		19362	*Pinus eliottii*	South Africa	Unknown		DQ396796	DQ393800
		19363	*Pinus eliottii*	South Africa	Unknown		DQ396797	DQ393801
O. dentifundum	115856	13017	*Quercus* sp.	Poland	T. Kowalski		AY495435	AY495446
	115790	13016	*Quercus* sp.	Hungary	C. Delatour		AY495434	AY495445
O. fusiforme	112912	9968	*Populus nigra*	Azerbaijan	D.N. Aghayeva	DQ294354	AY280481	AY280461
	112926	10565	*Larix decidua*	Austria	T. Kirisits		AY280484	AY280465
O. lunatum	112928	10564	*Larix decidua*	Austria	T. Kirisits	DQ294355	AY280486	AY280467
	112927	10563	*Carpinus betulus*	Austria	T. Kirisits		AY280458	AY280465
O. nigrocarpum	638.66	651	*Pseudotsuga menziesii*	USA	R.W. Davidson	DQ294356	AY280490	AY280480
	637.66	560	*Abies* sp.	USA	R.W. Davidson		AY280489	AY280479
O. palmiculminatum		20677	*Protea repens*	J. S. Marais Park, South Africa	F. Roets	DQ316143	DQ316191	DQ821543
		23049	*Trichouropoda* sp. from *P. repens*	J. S. Marais Park, South Africa	F. Roets	DQ821525	DQ821563	DQ821550
		23052	*Trichouropoda* sp. from *P. repens*	J. S. Marais Park, South Africa	F. Roets	DQ821526	DQ821566	DQ821574
		20693	*Protea repens*	J. S. Marais Park, South Africa	F. Roets		DQ316192	DQ821544
		23048	*Trichouropoda* sp. from *P. repens*	J. S. Marais Park, South Africa	F. Roets	DQ821527	DQ821565	DQ821549
		20694	*P. repens*	J. S. Marais Park, South Africa	F. Roets	DQ316144		DQ821546
		20695	*P. repens*	J. S. Marais Park, South Africa	F. Roets			DQ821545
		20696	*P. repens*	J. S. Marais Park, South Africa	F. Roets			DQ821548
		20697	*P. repens*	J. S. Marais Park, South Africa	F. Roets			DQ821547
		23053	*Trichouropoda* sp. from *P. repens*	J. S. Marais Park, South Africa	F. Roets	DQ821524	DQ821564	DQ821575

TABLE I. Continued

Species identity	Isolate no.		Host	Geographical origin	Collector	GenBank accession no.		
	CBS	CMW				LSU	ITS	B-tubulin
O. phasma		20676	*Protea laurifolia*	J. S. Marais Park, South Africa	F. Roets		DQ316219	DQ316181
		20698	*Protea laurifolia*	Giftberg top, South Africa	F. Roets	DQ316152	DQ316222	DQ316184
		20684	*Protea laurifolia*	Citrusdal, South Africa	F. Roets			DQ821540
		20676	*Protea laurifolia*	J. S. Marais Park, South Africa	F. Roets	DQ316151		DQ821541
		26	*Proctolaelaps vandenbergi from P. neriifolia*	Jonkershoek, SW Cape	F. Roets	DQ821535		
O. protearum	116654	1107	*Protea caffra*	Irene, South Africa	M.J. Wingfield	DQ316145	DQ316201	DQ316163
	116568	1102	*Protea caffra*	Irene, South Africa	M.J. Wingfield	AF221014		
	116567	1103	*Protea caffra*	Irene, South Africa	M.J. Wingfield		DQ316203	DQ316165
O. splendens	116569	872	*Protea caffra*	Unknown	M.J. Wingfield	AF221013	DQ316215	DQ296071
		20675	*Protea caffra*	George, South Africa	F. Roets		DQ316205	DQ316167
		20679	*Protea caffra*	JS Marais Park, SW Cape	F. Roets	DQ316150		
		23050	*Trichouropoda* sp. from *Protea repens*	JS Marais Park, SW Cape	F. Roets	DQ821534		
O. stenoceras		11192	Sapwood	New Zealand	R. Farrell		AY280492	AY280474
		2344	*Eucalyptus smithii*	South Africa	G.H.J. Kemp		AY280491	AY280472
	237.32	3202	*Pinus* sp.	Norway	H. Robak	DQ294350	AF484462	AY280471
Sporothrix sp. (unknown) [=*S. variecibatus* sp. nov.]	121962	2543	*Eucalyptus* sp.	Stellenbosch, South Africa	P.W. Crous		DQ821567	DQ821572
	121961	23051	*Trichouropoda* sp. from *Protea repens*	Stellenbosch, South Africa	F. Roets	DQ821537	DQ821568	DQ821539
	121960	23060	*Protea longifolia*	Kleinmond, South Africa	F. Roets		DQ821569	DQ821573
S. inflate	239.68	12572	Soil	Germany	W. Gams	DQ294351	AY495426	AY495445
	841.73		Wood	Chile	J. Grinbergs		AY495431	AY495442
S. schenckii	117440	7612	Human	South Africa	H. Vismer		AY280494	AY280476
	117842	7614	Human	South Africa	H. Vismer	DQ294352	AY280495	AY280477
		7615	Human	South Africa	H. Vismer		AY280496	AY280478

informative structure were made in all the investigated cultures and means (± standard deviation) calculated.

Mycelium-covered agar disks (5 mm diam) were excised from actively growing 1 wk old cultures of three different isolates of each of *O. palmiculminatum*, *O. gemellus* and *S. variecibatus*. These disks were transferred to the centers of fresh dishes containing 20 mL 2% MEA. The plates were incubated at 5–35 C with 5 C intervals for 2 d in the dark, after which colony diameters were determined. The procedure was repeated after an additional 8 d of growth in the dark. Both the mean diameter of additional growth (two measurements per replicate) and the mean growth diameter (± standard deviation) for each test species (three replicates) were calculated. Tolerance of these species to varying concentrations of cycloheximide (0.05, 0.1, 0.5, 1.0 and 2.5 g/L) was determined as described by Roets et al (2006) after 10 d of growth in the dark at 25 C.

Growth rates of *O. gemellus* and *S. variecibatus* on different concentrations of cycloheximide and varying temperatures were compared statistically to those of *O. palmiculminatum*. This was done to differentiate between these two species, which were found to be morphologically similar. A one-way analysis of variance (ANOVA) was used to analyze the data in the Statistica 7 (Statsoft Corp., Tulsa, Oklahoma) software package with Sigma-restricted parameterization. Significant differences between the growth rates of these fungal species are reported when $P \leq 0.05$.

DNA isolation, amplification and sequencing.—Genomic DNA from fungal mycelium was extracted with a Sigma GenElute™ plant genomic DNA miniprep kit (Sigma-Aldrich Chemie CMBH, Steinheim, Germany) according to the manufacturer's instructions. For amplification and sequencing of the nuclear large subunit (LSU) 28S rDNA region, the primers LROR and LR5 (White et al 1990) were used. The primers ITS1–F (Gardes and Bruns 1993) and ITS4 (White et al 1990) were used to amplify the ITS and 5.8S regions, while primers T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) were used to amplify the partial β-tubulin DNA regions.

Due to similarities in the DNA sequences of *O. palmiculminatum* and *O. gemellus*, β-tubulin gene fragments from selected isolates of these two species and *O. phasma*, *O. splendens*, as well as *S. variecibatus*, also were amplified with the primers T1 (O'Donnell and Cigelnik 1997) and Bt2b. This was done to obtain longer fragments of this gene region for comparisons. The extended β-tubulin dataset included the introns 2, 3 and 5 (amplified with primers T1 and Bt2b), while only intron 5 was amplified with the primer set T10 and Bt2b. PCR reaction mixtures and conditions for amplification of all gene regions followed the methods described by Roets et al (2006).

All amplified PCR products were cleaned with the Wizard® SV gel and PCR clean-up system (Promega, Madison, Wisconsin) following the manufacturer's instructions. Purified fragments were sequenced with the respective PCR primers and the Big Dye™ Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, California). The fragments were analyzed on an ABI PRISIM™ 3100 Genetic Analyzer (Applied Biosystems, Foster City, California).

Phylogenetic analyses.—Sequence data obtained in the laboratory were compared with sequence data acquired from GenBank for all known *Protea*-associated and various non-*Protea*-associated *Ophiostoma* spp. (TABLE I). Sequences were aligned with the Clustal X (1.81) software package.

28S ribosomal DNA region. A heuristic search with the Phylogenetic Analysis Using Parsimony (PAUP), v.4.0 beta 10 software package (Swofford 2000) was performed with tree-bisection-reconnection (TBR) branch swapping active. Characters were treated as unweighted. Starting trees were obtained through stepwise addition and resulting trees were combined into a consensus tree. One tree was saved per replicate to aid optimal searching of tree space. A total of 1000 bootstrap replicates (Felsenstein 1985) were performed with the fast-stepwise addition option active to estimate confidence levels.

Distance analysis was performed with the neighbor joining algorithm (Saitou and Nei 1987) in PAUP. The evolutionary model GTR + I + G (proportion of invariable sites at 0.7012 and the rates for variable sites following a gamma distribution with shape parameter of 1.0849) was selected with Modeltest 3.06 based on Akaike information criteria (Posada and Crandall 1998). Statistical support for nodes obtained by distance analysis was determined by 1000 bootstrap replicates using the TBR algorithm.

Bayesian analysis was performed with the GTR + I + G (shape parameter with four rate categories) model and the Markov chain Monte Carlo approach in the software package MrBayes v.3.1.1 (Ronquist and Huelsenbeck 2003). All parameters were inferred from the data. Two independent Markov chains of 1000 000 generations each (sample frequency of 50) were initiated from a random starting tree. The first 20 000 generations were discarded as burn-in and the remaining trees were pooled into a 50% majority rule consensus tree.

ITS, β-tubulin and extended β-tubulin datasets. *Ophiostoma nigrocarpum* (R.W. Davidson) de Hoog was chosen as outgroup based on results of Zipfel et al (2006), Roets et al (2006) and of analysis of the 28S ribosomal DNA dataset. Compatibility of the ITS and the β-tubulin (nonextended) datasets was tested with a SH test (Shimodaira and Hasegawa 1999) before combining them into a single dataset.

For neighbor joining analysis of the ITS, β-tubulin and extended β-tubulin datasets, evolutionary models for analysis again were determined with Modeltest 3.06. The selected evolutionary model for the combined dataset was GTR + I + G (proportion invariable sites 0.4598 and rates for variable sites following a gamma distribution with shape parameter of 0.5207). For distance analysis of the extended β-tubulin dataset the selected evolutionary model was HKY + I (proportion invariable sites 0.5326). Trees again were constructed with PAUP, using the neighbor joining tree-building algorithm (Saitou and Nei 1987). Statistical support for nodes was determined by 1000 NJ bootstrap replicates.

Data analyzed with Bayesian inference followed the methods outlined above. The general time-reversal model

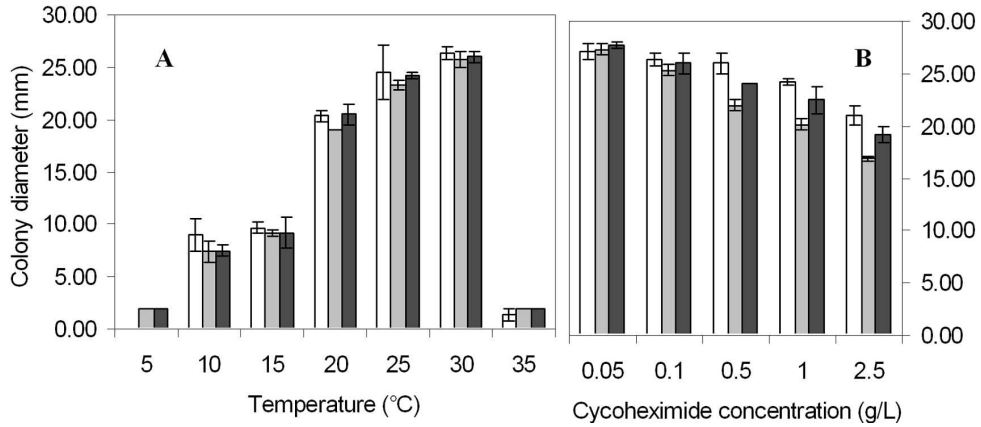


FIG. 1. Comparison of mean growth on MEA (three isolates per tested species, ± standard deviation) of *O. palmiculminatum* (gray bars), *Ophiostoma gemellus* (white bars) and *Sporothrix variecibatus* (black bars). A. held at different temperatures (grown 8 d in the dark). B. grown on different concentrations of cycloheximide (25 C for 10 d in dark).

of DNA substitution (Tavare 1986) with rate variation (four rate classes) and invariant sites was selected for these analyses. The first 1000 burn-in trees were discarded, and the remaining trees from both runs were pooled into a 50% majority rule consensus tree.

RESULTS

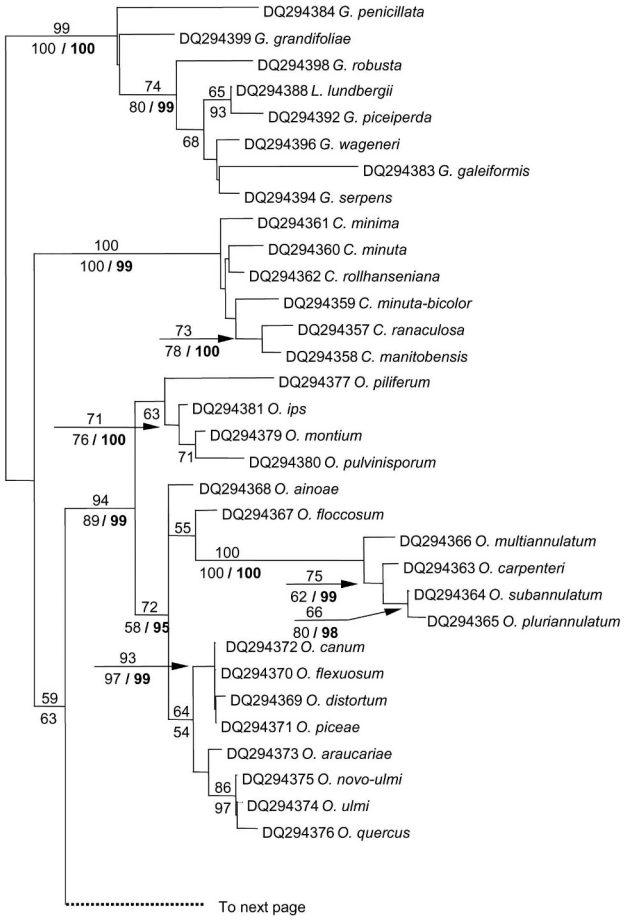
Morphology and growth in culture.—Roets et al (2007) showed that isolates obtained from mites collected from *Protea* infructescences could be divided into five morphological groups. Three of these groups are consistent with descriptions of the anamorphs of *O. palmiculminatum*, *O. phasma* and *O. splendens* respectively. The remaining two groups represented isolates of *O. gemellus* and *S. variecibatus* treated in this study. Differences in morphology between the anamorphs of *O. palmiculminatum* and *O. gemellus* were only slight and mostly related to size. For instance the length of the denticles of *O. gemellus* (ca. 2 μm) is usually twice as long as those of *O. palmiculminatum* (ca. 1 μm). The most reliable distinction however is the presence of clavate conidia in *O. palmiculminatum* while *O. gemellus* mostly produces c-shaped conidia. These differences were consistent between isolates representing the two species. *Sporothrix variecibatus* was morphologically different from the *Sporothrix* states of all known *Protea*-associated *Ophiostoma* spp.

After their initial isolation two isolates of *O. gemellus* (CBS numbers 121957 and 121959) formed mature ascomata on the MEA after 3 mo of growth at 25 C. Teleomorph structures of this species thus could be included in the morphological assessments. Subsequent subcultures using ascospore masses failed to produce mature ascomata. Comparisons of the morphology of *S. variecibatus* and other *Ophiostoma* spp. were based on anamorph structures only because no teleomorph structures of this taxon were found.

Cultures of *O. palmiculminatum*, *O. gemellus* and *S. variecibatus* grew optimally at 30 C (FIG. 1a) on MEA. The mean colony diameter of the *O.* gemellus was 26.3 mm (± 0.6), while *S. variecibaus* had a colony diameter of 26 mm (± 0.5) at this temperature after 8 d of growth in the dark. Under these conditions the mean colony diameter at the optimum growth temperature for *O. palmiculminatum* was 25.7 mm (± 0.8). Comparisons of growth rate between *O. gemellus* and *O. palmiculminatum* at different temperatures on MEA revealed no significant differences. Both had similar growth at the different temperature intervals, with peaks at 30 C, after which a rapid decline was observed to 35 C (FIG. 1a).

All species were tolerant to relatively high levels of the antibiotic cycloheximide in the growth media. The growth rate (as measured by mean colony diameter) of *O. gemellus* declined from 27.2 mm (± 0.8) on 0.05 g/L to 21 mm (± 0.9) on 2.5 g/L cycloheximide after 10 d (FIG. 1b). The growth rate of *S. variecibatus* declined from 27.7 mm (± 0.3) on 0.05 g/L to 19.2 mm (± 0.8) on 2.5 g/L cycloheximide after 10 d (FIG. 1b). Growth rate for *O. palmiculminatum* declined from 27 mm (± 1) on 0.05 g/L to 17 mm on 2.5 g/L cycloheximide after 10 d (FIG. 1b).

The difference in growth between *Ophiostoma gemellus* and *O. palmiculminatum* on the different cycloheximide concentrations (FIG. 1b) was highly significant ($F = 124.16$, $P < 0.0001$). In addition the two taxa also reacted significantly different to changes in cycloheximide concentration ($F = 15.23$, $P < 0.0001$). *Ophiostoma palmiculminatum* was more sensitive to this antibiotic than the isolates of *O. gemellus*.



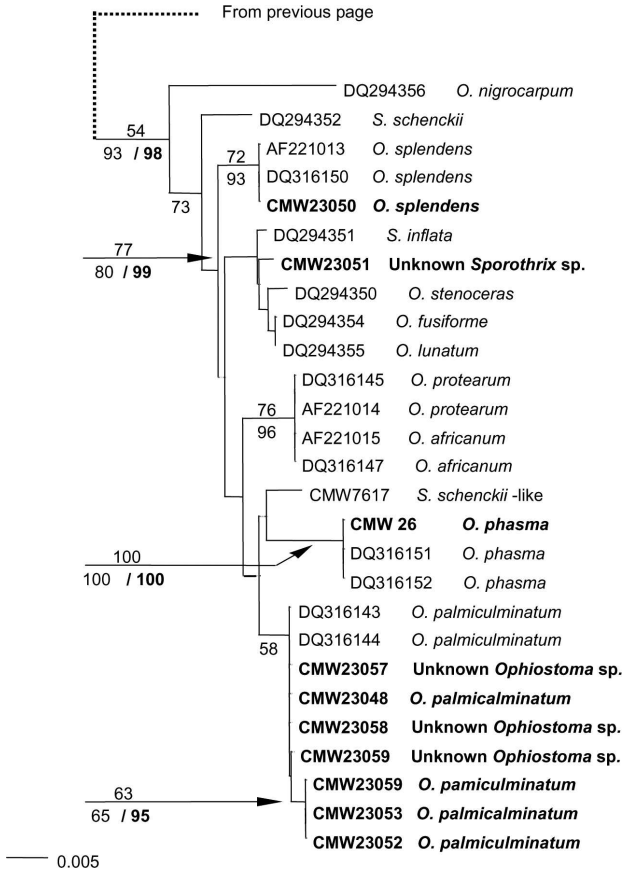


FIG. 2. Continued.

FIG. 2. Neighbor joining tree derived from the 28S rDNA dataset. Values above nodes indicate parsimony-based bootstrap values obtained by 1000 replicates. Values below nodes indicate bootstrap values (1000 replicates) obtained from neighbor joining analysis. Values in boldface below nodes represent posterior probabilities (%) obtained with Bayesian inference. Isolates obtained from mites in this study are indicated in bold typeface.

DNA isolation, amplification and sequencing.—Amplified fragments obtained with primers LROR and LR5 were ca. 700 bp long. Amplification of extracted genomic DNA with the primers ITS1–F and ITS4 resulted in fragments ca. 550–600 bp long. DNA fragments ca. 500–560 bp long were amplified with the primers T10 and Bt2b. Substantially longer fragments (ca. 700–800 bp) were obtained when amplifying the extracted genomic DNA with primer pairs T1 and Bt2b.

Phylogenetic analyses.—28S ribosomal DNA dataset. The aligned 28S ribosomal DNA dataset consisted of 706 characters of which the numbers of parsimony informative, parsimony uninformative and constant characters were respectively 98, 29 and 579. Analysis with the parsimony algorithm yielded 67 equally most parsimonious trees of 287 steps long. The consistency index (CI) was 0.4321, while the retention index (RI) was 0.8378. Phylogenetic reconstruction of the genus based on LSU sequences indicated that all isolates from mites represented *Ophiostoma* spp., even though sexual structures were not observed for most of the isolates (FIG. 2). These analyses confirmed that *O. palmiculminatum*, *O. splendens* and *O. phasma* (FIG. 2) were collected from *Tarsonemus* cf. sp. *A.*, *P. vandenbergi* and the *Trichouropoda* sp. (Roets et al 2007). The phylogenetic reconstruction also revealed that the isolate of *S. variecibatus* (CBS 23051) from the *Trichouropoda* sp. mite, resided in a clade distinct from any of the *Ophiostoma* spp. known from *Protea* infructescences (FIG. 2).

No differences were found in comparisons among large subunit data of *O. palmiculminatum* and the three isolates from *Tarsonemus* cf. sp. B collected from *P. caffra* (FIG. 2). Isolates representing *O. palmiculminatum* and those of *O. gemellus* however were distinct based on morphological comparisons. Conidia of *O. palmiculminatum* are clavate (Roets et al 2006), whereas c-shaped conidia were formed by isolates of *O. gemellus*.

ITS, ß-tubulin and extended ß-tubulin datasets. Alignment of the amplified sequence fragments of the

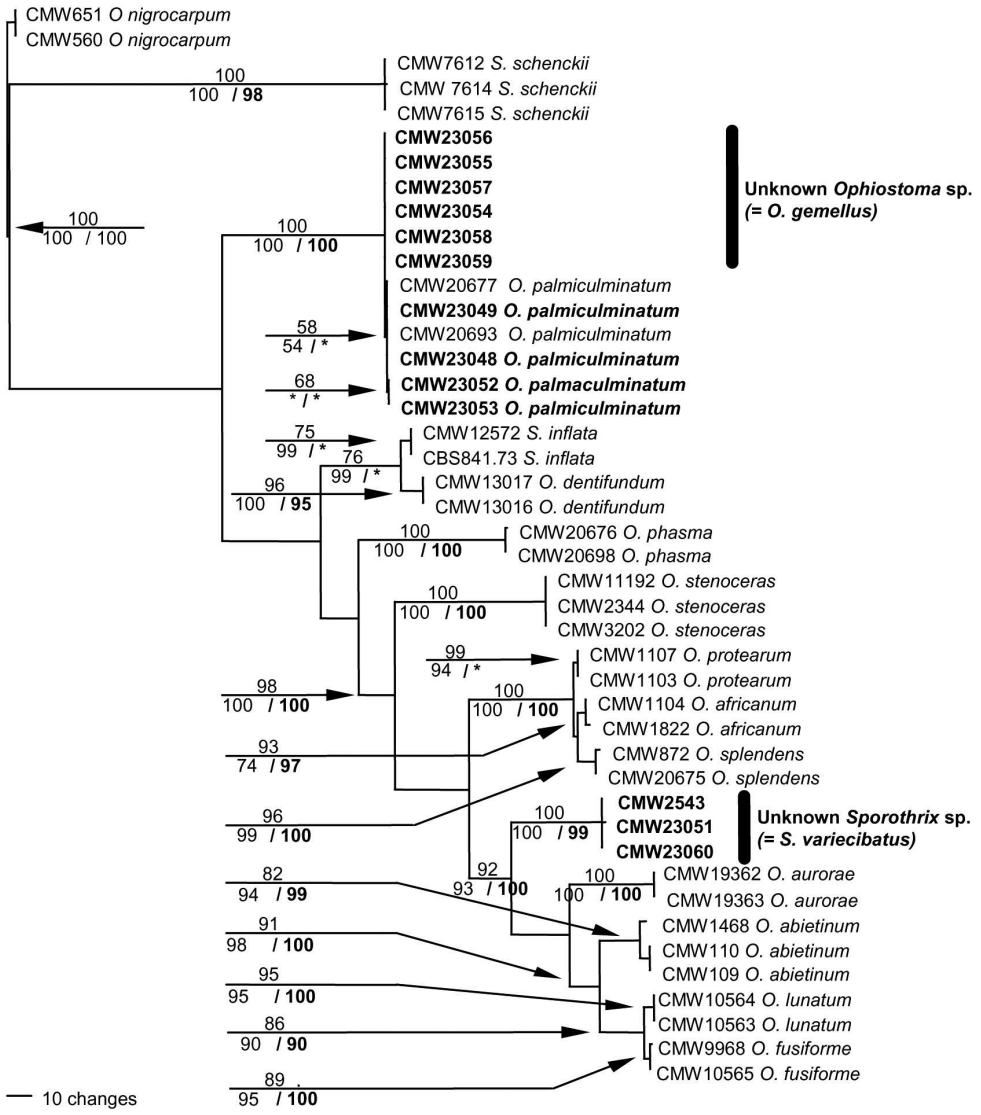


FIG. 3. One of 70 equally parsimonious trees obtained for the combined ITS and β-tubulin dataset. Values above nodes indicate bootstrap values (1000 replicates) of neighbor joining analysis obtained with the GTR + I + G parameter model (G = 0.5207). Values below nodes indicate parsimony-based bootstrap values (1000 replicates). Values in boldface represent confidence values (posterior probabilities as percentage) obtained with Bayesian inference. (* = value below 50 [= value below 95% for Bayesian analysis]). Isolates collected in this study are indicated in boldface.

respective ITS, β-tubulin and extended β-tubulin gene regions resulted in datasets of 603 273 and 545 characters. Numbers of potentially parsimony informative, parsimony uninformative and constant characters were 171, 0 and 432 for ITS; 108, 1 and 164 for β-tubulin; and 147, 34 and 364 for the extended β-tubulin datasets.

The ITS and β-tubulin datasets (excluding extended β-tubulin data) were combined in spite of the outcome of the SH test ($P < 0.05$) because the observed differences between these were most likely the result of ambiguous alignment due to the variability of the β-tubulin intron areas of the various species. Placement of isolates of the various species of interest in this study in the trees resulting from phylogenetic analysis of the datasets for each separate gene region was similar. Combining datasets did not affect the grouping of terminal nodes of interest compared to the phylogenetic reconstructions using the separate datasets.

After alignment the combined dataset for the ITS and β-tubulin gene regions consisted of 876 characters. Numbers of potentially parsimony informative, parsimony uninformative and constant characters for the combined dataset were respectively 279, 1 and 596. Parsimony analysis of these data resulted in 70 equally most parsimonious trees 573 bp long and had a CI = 0.716 and RI = 0.923.

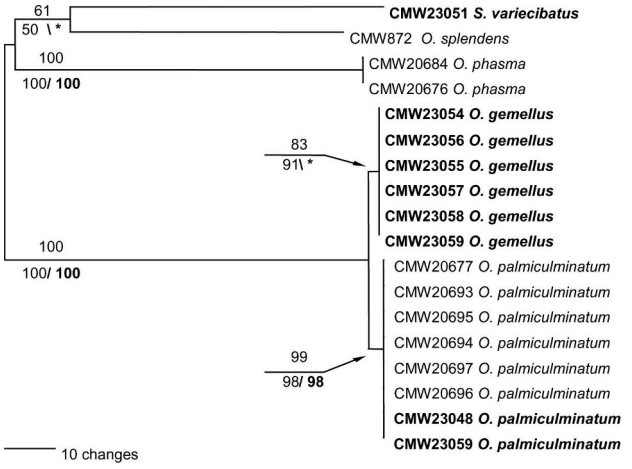


FIG. 4. The most parsimonious tree obtained for the extended β-tubulin dataset (including exons 2–5, partial exon 6 and introns 2, 3 and 5). Values above nodes indicate bootstrap values (1000 replicates) of neighbor joining analysis obtained with the HKY + I parameter model. Values below nodes indicate parsimony-based bootstrap values (1000 replicates). Values in bold typeface represent confidence values (posterior probabilities as percentage) obtained with Bayesian inference. (* = value below 95%.) Isolates collected in this study are indicated in boldface.

Isolates of *S. variecibatus* grouped with the isolate (CMW 2543) from *Eucalyptus* with strong support obtained by all three phylogenetic node support algorithms (FIG. 3). They formed a strongly supported monophyletic clade sister of *Ophiostoma abietinum* Marm. & Butin, *O. aurorae* X.D. Zhou & M.J. Wingf., *O. fusiforme* Aghayeva & M.J. Wingf. and *O. lunatum* Aghayeva & M.J. Wingf., deeply embedded within the phylogenetic reconstruction of the genus.

Analysis of the combined ITS and β-tubulin gene regions accentuated a close relationship between *O. gemellus* and *O. palmiculminatum* because they were separated by weak support with the three phylogenetic support algorithms (FIG. 3). Isolates of these two taxa grouped together into one well supported clade, suggesting an affinity between them. The phylogenetic difference between *O. palmiculminatum* and *O. gemellus* is better demonstrated by analyses of the sequence data for the extended β-tubulin gene region of these taxa. This dataset included eight isolates of *O. palmiculminatum*, two of *O. phasma*, one of *O. splendens*, six of *O. gemellus* and one of *S. variecibatus*. Parsimony analysis of this dataset resulted in one most parsimonious tree 244 bp long and had a CI = 0.955 and RI = 0.961 (FIG. 4).

Strong support values were attained for the divergence between the isolates representing *O. palmiculminatum* and *O. gemellus* when the data from the extended β-tubulin gene region was analyzed with both neighbor joining and parsimony bootstrap support algorithms (FIG. 4). The isolates representing these lineages were found to diverge in terms of 5 bp positions. These differences also were consistent for the two lineages. Strong Bayesian support values (posterior probabilities) were obtained for the monophyly of *O. palmiculminatum*, *O. phasma* and the clustering of *O. palmiculminatum* with *O. gemellus* (FIG. 4).

TAXONOMY

From the morphological comparisons and growth study data obtained, it was clear that the two mite-associated *Ophiostoma* spp. with *Sporothrix* anamorphs from *Protea* infructescences were different from any *Ophiostoma* described from this niche. These fungi also could be distinguished from previously described *Ophiostoma* spp. based on DNA comparisons. They therefore are newly described as follows:

Ophiostoma gemellus Roets, Z.W. de Beer & P.W. Crous., sp. nov. MycoBank MB511456 FIG. 5

Ophostomati palmiculminato simile, sed basi ascomatum latiore (70–270 μm), collo ascomatum breviore et crassiore (200–525 × 12–18 μm), hyphis ostiolaribus longioribus (32–42 μm) et conidiis curvatis differens.

Ascomata superficial on 2% MEA plates after 2 mo of growth at room temperature. *Ascomatal bases* globose, dark, 70–270 μm (176 ± 75) diam, without hyphal ornamentation. *Ascomatal necks* dark brown to black, 200–525 μm (430 ± 101) long, 40–50 μm (46 ± 4) wide at the base, 12–18 μm (15 ± 2) wide at the apex. Ten to thirteen *ostiolar hyphae* usually present, somewhat curved, hyaline to subhyaline, 32–42 μm (35 ± 3) long (FIG. 5a–c). *Asci* evanescent. *Ascospores* allantoid, one-celled, hyaline, sheaths absent, 3–5 μm (5 ± 1) long, 1–2 μm wide (FIG. 5c) collecting in a hyaline gelatinous droplet at the apex of the neck, remaining uncolored when dry.

Culture of the Sporothrix anamorph on MEA 24.5 μm (± 2.64) mm diam after 8 d at 25 C in the dark, white to cream, effuse, circular with an entire edge, surface smooth. Growth reduced at temperatures below and above the optimum of 30 C. *Hyphae* superficial on 2% MEA plates (FIG. 5d). Sporulation profuse on MEA. *Conidiogenous cells* 3–44 μm long, 1.5–2.5 μm wide, arising directly from hyphae and from 5–45 μm long aerial conidiophores, proliferating sympodially, hyaline (FIG. 5f–k) becoming denticulate. *Denticles* 0.5–2.5 μm (2 ± 0.5) long, usually in an apical crown of 5–12, sometimes in an extended zone 4–8 μm long, scattered, solitary or in nodes. *Conidia* holoblastic, hyaline, one-celled, clavate to strongly curved, smooth, thin-walled, 3–7 μm

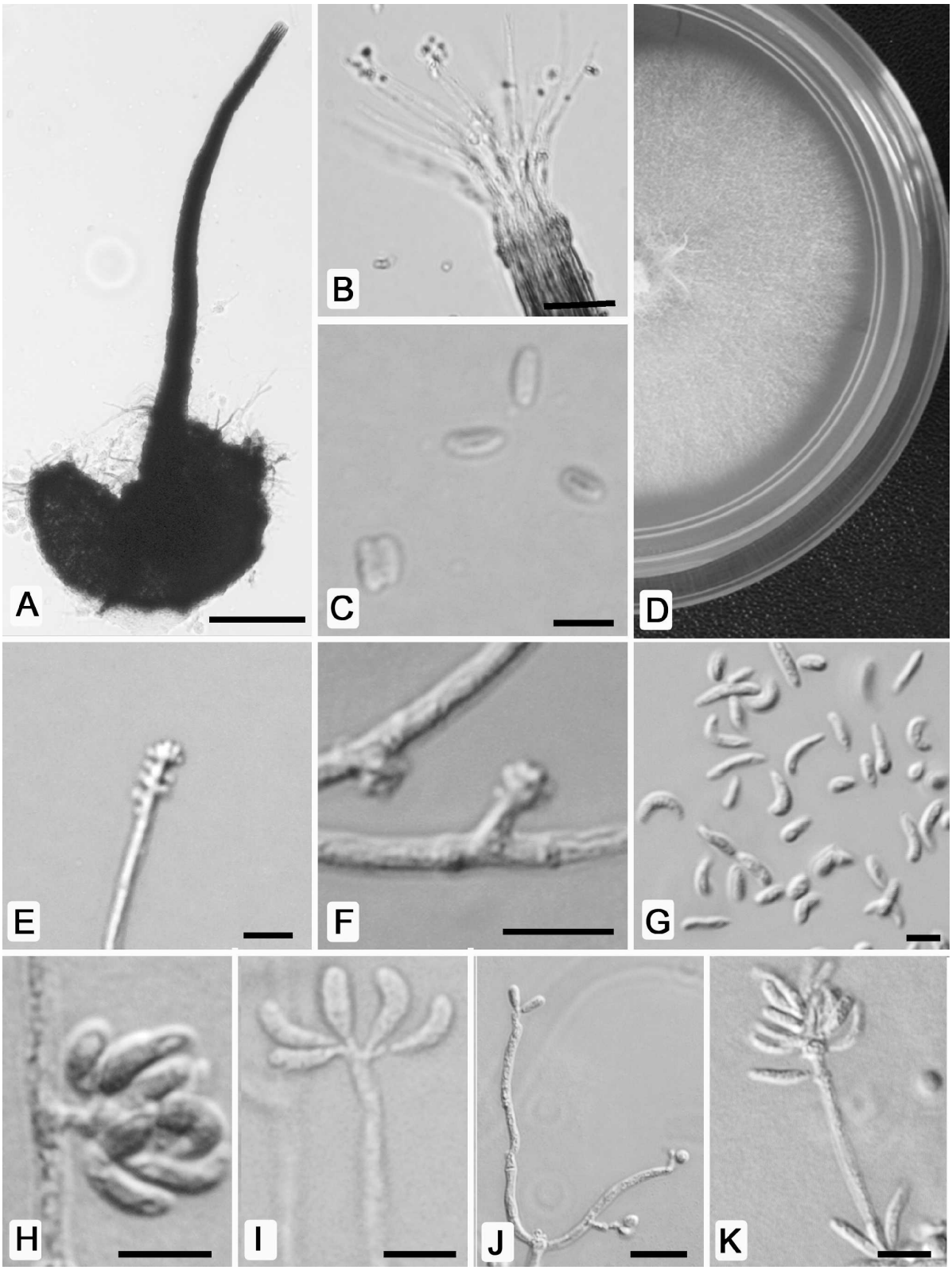


FIG. 5. Micrographs of *Ophiostoma gemellus* prov. nom. A. Ascoma produced on the surface of MEA agar after 3 mo of growth at 24 C. B. Ostiolar hyphae. C. Ascospores. D. Two-week-old colony of the *Sporothrix* anamorph on MEA. E. Conidiogenous cell on long conidiophore. F. Conidiogenous cells arising directly from hyphae. G. Conidia. H–K. Conidiogenous cells arising from hyphae and conidiophores of varying lengths. Bars: A = 100 µm, B = 10 µm, C–I = 5 µm, J = 10 µm, K = 5 µm.

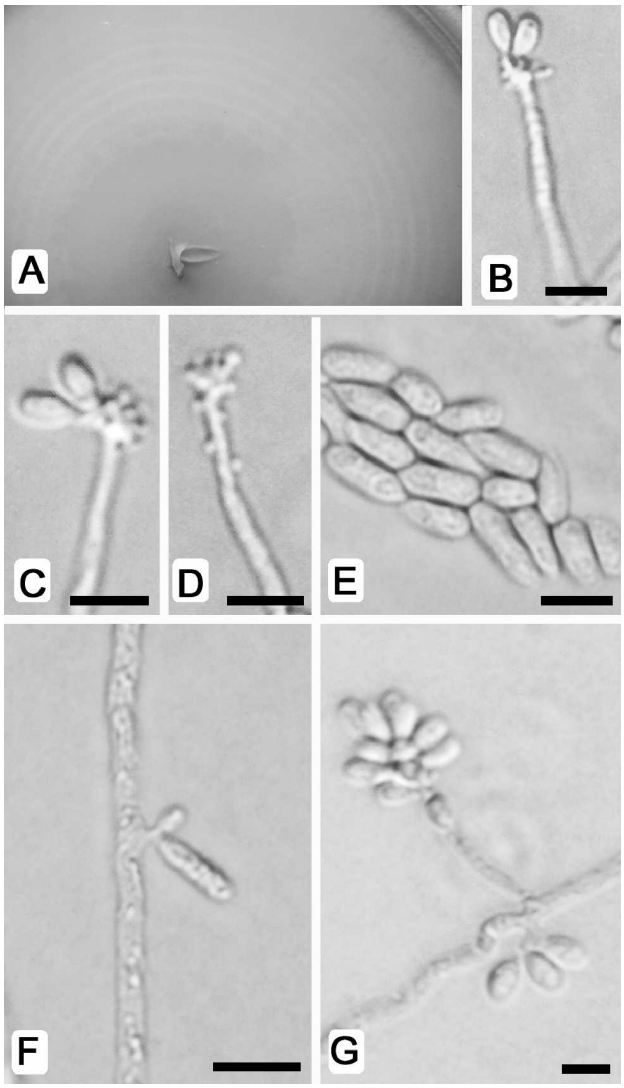


FIG. 6. Micrographs of *Sporothrix variecibatus* prov. nom. A. Two-week-old colony on MEA B–D. Conidiogenous cells. E. Conidia. F–G. Conidia arising directly from hyphae and conidiophores of various lengths. Bars = 5 μm.

(5 ± 2) long and 2–3.5 μm (3) wide (FIG. 5e). Conidia formed singly but aggregate to form slimy masses.

Etymology: The epithet *gemellus* (*gemellus* = twin) refers to the close resemblance of the ascomata to its sister species *O. palmiculminatum*.

Specimens examined: SOUTH AFRICA. GAUTENG PROVINCE: Walter Sisulu National Botanical Garden. From the mite *Tarsonemus* sp. from within the infructescences of *P. caffra*, Apr 2005, *F. Roets*, (HOLOTYPE PREM 59836) (culture CMW 23057 = CBS 121959); GAUTENG PROVINCE: Walter Sisulu National Botanical Garden. From the mite *Tarsonemus* sp. from within the infructescences of *P. caffra*, Apr 2005, *F. Roets*, (PARATYPE PREM 59837) (culture CMW 23058); GAUTENG PROVINCE: Walter Sisulu National Botanical Garden. From the mite *Tarsonemus* sp. from within the infructescences of *P. caffra*, Apr 2005, *F. Roets*, (culture CMW 23059); GAUTENG PROVINCE: Walter Sisulu National Botanical Garden. Within *P. caffra* infructescences, May 2004, *F. Roets*, (culture CMW 23054 = CBS 121957); GAUTENG PROVINCE: Walter Sisulu National Botanical Garden. Within *P. caffra* infructescences, May 2004, *F. Roets*, (culture CMW 23056 = CBS 121958); GAUTENG PROVINCE: Walter Sisulu National Botanical Garden. Within *P. caffra* infructescences, May 2004, *F. Roets*, (CMW 23055).

Notes. Based on morphological characteristics *O. gemellus* is closely related to *O. palmiculminatum*. These nucleotide characters are diagnostic (presented as the gene followed by the nucleotide position in the gene in brackets) of *O. gemellus* as compared to *O. palmiculminatum*: internal transcribed spacer 1 of the nuclear encoded rDNA, position 188 (C instead of T); β-tubulin gene intron 2, positions 59 (G instead of A), 74 (A instead of C) and 115 (C instead of A); β-tubulin gene intron 3, positions 31 (C instead of A) and 34 (A instead of C).

Sporothrix variecibatus Roets, Z.W. de Beer & P.W. Crous, sp. nov. MycoBank MB511457 FIG. 6

Anamorphe Ophiomatis aurorae similis, sed conidiis minoribus, 3–7 × 2–3 μm, differens.

Ascomata not observed. Cultures of the *Sporothrix* sp. on MEA 24.17 mm (± 0.29) diam after 8 d at 25 C in the dark, white to cream, effuse, circular with an entire edge, surface smooth. Growth reduced at temperatures below and above the optimum of 30 C. *Hyphae* superficial on 2% MEA plates (FIG. 6a). Sporulation profuse on MEA. *Conidiogenous cells* 5–20 μm long, 1.5–2 μm wide, arising directly from hyphae or from short (19 μm ± 6) aerial conidiophores, proliferating sympodially, hyaline (FIG. 6b–e) becoming denticulate. *Denticles* 1–2 μm (1.5 ± 1) long, usually in an apical crown of 9–16, sometimes in an extended zone 5–10 μm long. *Conidia* holoblastic, hyaline, one-celled, clavate, smooth, thin-walled, 3–7 μm (6 ± 2) long and 2–3 μm (2) wide (FIG. 6f). *Conidia* forming singly, aggregating to form slimy masses.

Teleomorph: not observed, phylogenetically *Ophiostoma*.

Etymology: The epithet *variecibatus* (*varie* = diverse, *cibatus* = food) refers to the taxonomically diverse host range from which isolates of this species were collected.

Specimens examined: SOUTH AFRICA. WESTERN CAPE PROVINCE: Stellenbosch, Jan S. Marais Park. From *Oodinychys* sp. mite associated with *P. repens*, Jul 2004, *F. Roets*, (HOLOTYPE PREM 59838) (culture CMW 23051 = CBS 121961); WESTERN CAPE PROVINCE: Kleinmond district. Within the infructescences of *P. longifolia*, Jul 2004, *F. Roets*, (PARATYPE PREM 59839) (culture CMW 23060 =

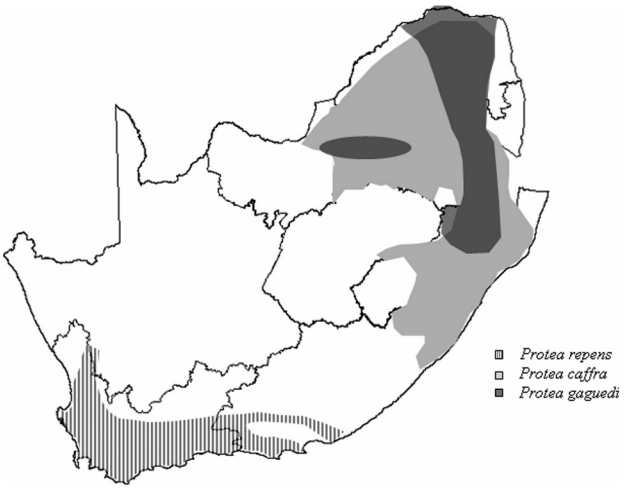


FIG. 7. Distribution of *Ophiostoma* host *Protea* species in South Africa.

CBS 121960); WESTERN CAPE PROVINCE: Stellenbosch district. From the leaf litter of *Eucalyptus* sp., Apr 1993, *P.W. Crous*, (PARATYPE PREM 59840) (culture CMW 2543 = CBS 121962).

DISCUSSION

This study led to the discovery of two new ophiostomatoid species associated with the infructescences of *Protea* spp. in South Africa. *Ophiostoma gemellus* is known from both the teleomorph and anamorph states. In contrast *Sporothrix variecibatus* is recognized as a new species of *Ophiostoma* based on its phylogenetic placement in this genus but in the absence of a teleomorph. Description of these two new species brings to seven the species of *Ophiostoma* known from *Protea* hosts in South Africa. These fungi typically are restricted to the infructescences of serotinous members of the host genus and have a wide distribution throughout South Africa. The newly described *O. gemellus* and *S. variecibatus* were known previously only from mites collected from *Protea* plants (Roets et al 2006). In this study they were also collected from the infructescences of *Protea* spp. from which the mites had been collected.

From both the DNA sequence comparisons and morphological characters, it is clear that *O. gemellus* is closely related to its sister species *O. palmiculminatum*. This is indicated by the low support values obtained for the separation of these taxa using parsimony, Bayesian, and neighbor joining analyses of the combined ITS and β-tubulin dataset. Phylogenetically the species were separated however by analyses of the extended β-tubulin dataset. High support values were obtained when analyzing this data with both the parsimony and neighbor joining algorithms. Low support for the monophyly of *O. gemellus* using Bayesian inference likely was due to the long branches leading to the *O. palmiculminatum*-*O. gemellus* clade and outgroups.

Morphological differences between the anamorphs of *O. palmiculminatum* and *O. gemellus* include the conidial shape with the conidia of *O. palmiculminatum* being clavate while *O. gemellus* forms c-shaped conidia in culture. Morphologically the teleomorphs also differ slightly, most notably in the length of ostiolar hyphae. The ostiolar hyphae of *O. gemellus* are about twice as long as those of *O. palmiculminatum*. Physiologically the two species differ markedly in their responses to different cycloheximide concentrations. The most obvious distinction between these two species however is their different host species. *Ophiostoma gemellus* is known only from *P. caffra* while *O. palmiculminatum* apparently is specific to *P. repens*.

Sporothrix variecibatus appears to be related to *O. stenoceras*, which has been reported globally from wood and soil (de Beer et al 2003), to hardwood infecting species such as *O. fusiforme* and *O. lunatum* (Aghayeva et al 2005), and to the conifer bark-beetle associates *O. abietinum* and *O. aurorae* (Zhou et al 2006). Our data suggest that the closest relative of *S. variecibatus* is *O. aurorae*, which recently was described from bark beetles infesting *Pinus* spp. in the Mpumalanga Province of South Africa (Zhou et al 2006). Morphologically the *Sporothrix* anamorph of *O. aurorae* and *S. variecibatus* are similar. They closely resemble other species in the *O. stenoceras*-complex (de Beer et al 2003), and in the absence of a teleomorph these species are morphologically almost indistinguishable. However they can be distinguished from other species in the complex by their swollen clavate conidia, with those of *O. aurorae* being slightly larger than conidia produced by *S. variecibatus*. Comparisons of ITS and partial β-tubulin sequence data also showed that *O. aurorae* and *S. variecibatus* are distinct species and that they differ from other similar species in the *O. stenoceras*-complex, for which data was available.

Sporothrix variecibatus represents the first known example of an *Ophiostoma* sp. associated with *Protea* that has been isolated from material of an unrelated host, in this case the leaf litter of an exotic *Eucalyptus* sp. The known *Protea* hosts of this fungus include *P. repens* from J.S. Marais Park in Stellenbosch and *P. longifolia* from a site in the Kleinmond district. *Eucalyptus* and *P. repens* plants were found growing together close to the site where *S. variecibatus* was isolated from *Eucalyptus* in J.S. Marais Park. At this stage the data are insufficient to draw clear conclusions on whether *S. variecibatus* shifted from native

Protea spp. hosts to the *Eucalyptus* litter environment or vice versa. Because *S. variecibatus* also was isolated from a *Trichouropoda* mite, we believe that this mite could have aided the movement of the fungus from *Protea* to *Eucalyptus* leaf litter.

Ophiostoma palmiculminatum and *O. gemellus* also were isolated from mites associated with the infructescences of their respective hosts. Although *O. palmiculminatum* and *O. gemellus* represent sister species, the respective mite species associated with them are distantly related. *Ophiostoma gemellus* was isolated from a *Tarsonemus* sp. (Tarsonemidae), while *O. palmiculminatum* was isolated from a *Trichouropoda* sp. (Uropodidae). *Trichouropoda* mites have not been observed from the infructescences of *P. caffra* but are common within the infructescences of *P. repens* (pers obs). In contrast *Tarsonemus* sp. has been recorded from *P. repens* infructescences (Roets et al 2007), but no isolates of *O. palmiculminatum* were collected from *Tarsonemus* sp. mites in that study. The speciation event that resulted in the separation of *O. gemellus* and *O. palmiculminatum* thus might have been driven both by differences in host species and a switch in vectors.

The geographic distribution of the respective hosts of the sister species *O. gemellus* (*P. caffra*) and *O. palmiculminatum* (*P. repens*) are disjunct, with *P. caffra* occurring in the northern and eastern parts South Africa while *P. repens* is confined to the Western Cape Province of South Africa (FIG. 7). Although not in the same monophyletic lineage as *O. gemellus* and *O. palmiculminatum*, the sister species *O. protearum* and *O. africanum* (from *P. caffra* and *P. gaguidi* respectively) show the same pattern of north-south disjunction with their closest relative *O. splendens*. The *Protea* hosts of *O. protearum* and *O. africanum* are restricted to northeastern South Africa, while their sister species *O. splendens* occurs only on *Protea* spp. (including *P. repens*) in southwestern South Africa (FIG. 7). The northern and southern *Protea*-rich areas of South Africa are separated by the dry central Karoo region that effectively is devoid of *Protea* spp. A repeated pattern of speciation in the *Protea*-associated members of *Ophiostoma* thus appears to have been driven by a combination of geographical separation and accompanying host switches.

The *Ophiostoma* spp. associated with *Protea* infructescences are morphologically similar. This morphological uniformity has rendered molecular phylogenetic analysis essential to the identification and taxonomic placement of these fungi. Additional cryptic species are likely to be discovered as isolates from a wider geographical range and a wider host range are included. The close morphological similarity between all *Protea*-associated Ophiostomatoid fungi (*Ophiostoma* and *Gondwanamyces* spp.) might be ascribed to similarities in ecological and evolutionary pressures (e.g. a shared arthropod-vectored mode of spore dispersal). This is supported by the recent confirmation that at least four *Protea*-associated *Ophiostoma* spp. and one *Gondwanamyces* sp. are associated strongly with mite species present on their host plants (Roets et al 2007, Roets unpubl data). Future studies will focus on defining the vectors of the remaining *Protea*-associated *Ophiostoma* spp. and clarifying the number of species involved in these multi-organism interactions.

ACKNOWLEDGMENTS

We thank the National Research Foundation (NRF) and the NRF/DST Centre of Excellence in Tree Health Biotechnology (CTHB) for financial support, as well as colleagues at the Forestry and Agricultural Biotechnology Institute (FABI) and the Centraalbureau voor Schimmelcultures (CBS) for making cultures available for study. We also thank the Western Cape Nature Conservation Board for issuing the necessary collecting permits. Dr Walter Gams (CBS) is acknowledged for providing the Latin diagnoses.

LITERATURE

Aghayeva DN, Wingfield MJ, Kirisits T, Wingfield BD. 2005. *Ophiostoma dentifundum* sp. nov. from oak in Europe, characterised using molecular phylogenetic data and morphology. Mycol Res 109:1127–1136.

Ayres MP, Wilkens RT, Ruel JJ, Vallery E. 2000. Fungal relationships and the nitrogen budget of phloem-feeding bark beetles (Coleoptera: Scolytidae). Ecology 81:2198–2210.

Barras SJ, Perry TJ. 1975. Interrelationships among microorganisms, bark or ambrosia beetles, and woody host tissue: an annotated bibliography, 1956–1974. USDA Forest Service Gen. Tech. Rep. SO-10.

Beaver RA. 1989. Insect-fungus relationships in the bark and ambrosia beetles. In: Wilding N, Collins NM, Hammond PM, Webber JF, eds. Insect-fungus interactions. London: Academic Press. p 121–143.

Berryman AA. 1989. Adaptive pathways in Scolytid-fungus associations. In: Wilding N, Collins NM, Hammond PM, Webber JF, eds. Insect-fungus interactions. London: Academic Press. p 121–143.

Bridges JR, Moser JC. 1983. Role of two phoretic mites in transmission of bluestain fungus, *Ceratocystis minor*. Ecol Entomol 8:9–12.

———, ———. 1986. Relationship of phoretic mites (Acari: Tarsonemidae) to the bluestaining fungus, *Ceratocystis minor*, in trees infested by southern pine beetle (Coleoptera: Scolytidae). Environ Entomol 15:951–953.

Cassar S, Blackwell M. 1996. Convergent origins of ambrosia fungi. Mycologia 88:596–601.

De Beer ZW, Harrington TC, Vismer HF, Wingfield BD, Wingfield MJ. 2003. Phylogeny of the *Ophiostoma*

stenoceras-Sporothrix schenckii complex. Mycologia 95: 434–441.

———, Wingfield MJ, Kemp GHJ. 1995. First report of *Ophiostoma querci* in South Africa. Proceedings of the 32nd Annual Congress for the South African Society for Plant Pathology. S Afr J Sci 91:VI.

———, Witthuhn RC, Britz H, Wingfield MJ, Wingfield BD. 1999. 18S rDNA sequences place *Ophiostoma* isolates from hardwoods in the southern hemisphere in the species *Ophiostoma stenoceras*. Proceedings of the 37th Annual Congress for the South African Society for Plant Pathology. S Afr J Sci 95:VI.

Felsenstein J. 1985. Confidence limits on phylogenetics: an approach using the bootstrap. Evolution 39:783–791.

Francke-Grosmann H. 1967. Ectosymbiosis in wood-inhabiting insects. In: Henry SM, ed. Symbiosis. Vol II. New York: Academic Press. p 171–180.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl Environ Microbio 61:1323–1330.

Harrington TC. 1987. New combinations in *Ophiostoma* of *Ceratocystis* species with *Leptographium* anamorphs. Mycotaxon 28:39–43.

Jacobs K, Wingfield MJ. 2001. *Leptographium* species: tree pathogens, insect associates and agents of blue-stain. St Paul, Minnesota: APS Press.

Kirisits T. 2004. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In: Lieutier F, Day KR, Battisti A, Grégoire JC, Evans H, eds. Bark and wood boring insects in living trees in Europe, a synthesis. The Netherlands: Kluwer Academic Press. p 1–55.

Klepzig KD, Moser JC, Lombardero MJ, Ayres MP, Hofstetter RW, Walkinshaw CJ. 2001a. Mutualism and antagonism: ecological interactions among bark beetles, mites and fungi. In: Jeger MJ, Spence NJ, eds. Biotic interactions in plant-pathogen associations. Cambridge: CAB International. p 237–267.

———, ———, ———, ———, Ayres MP. 2001b. Symbiosis and competition: complex interactions among beetles, fungi and mites. Symbiosis 30:83–96.

Lévieux J, Lieutier J, Moser JC, Perry TJ. 1989. Transportation of phytopathogenic fungi by the bark beetle *Ips sexdentatus* Boemer and associated mites. J Appl Entomol 108:1–11.

Linder HP. 2003. The radiation of the Cape flora, southern Africa. Biol Rev 78:597–638.

Malloch D, Blackwell M. 1993. Dispersal biology of the ophiostomatoid fungi. In: Wingfield MJ, Seifert KA, Webber JF, eds. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. St Paul, Minnesota: APS Press. p 195–206.

Marais GJ, Wingfield MJ. 1994. Fungi associated with infructescences of *Protea* species in South Africa, including a new species of *Ophiostoma*. Mycol Res 98: 396–374.

———, ———. 1997. *Ophiostoma protearum* sp. nov. associated with *Protea caffra* infructescences. Can J Bot 75:362–367.

———, ———. 2001. *Ophiostoma africanum* sp. nov., and a key to ophiostomatoid species from *Protea* infructescences. Mycol Res 105:240–246.

Moser JC. 1997. Phoretic mites and their hyperphoretic fungi associated with flying *Ips typographys japonicus* Niijima (Coleoptera: Scolytidae) in Japan. J Appl Entomol 121:425–428.

———, Perry TJ, Solheim H. 1989. Ascospores hyperphoretic on mites associated with *Ips typographus*. Mycol Res 93:513–517.

Münch E. 1907. Die Blaufäule des Nadelholzes. I–II. Naturwissenschaftliche Zeitschrifr für Land- und Forstwitschaft 5:531–573.

———. 1908. Die Blaufäule des Nadelholzes. III–IV. Naturwissenschaftliche Zeitschrifr für Land- und Forstwitschaft 6:32–47, 297–323.

Norris DM. 1979. The mutualistic fungi of xyleborine beetles. In: Batra LR, ed. Insect-fungus symbiosis. Sussex, UK: Halsted Press. p 53–63.

O'Donnell K, Cigelnik E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol Phylogenet Evol 7:103–116.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818.

Price TS, Doggett C, Pye JM, Holmes TP. 1992. A history of southern pine beetle outbreaks in the southeastern United States. Macon: Georgia Forestry Commission.

Rebelo T. 1995. Proteas of South Africa. Capetown: Fernwood Press.

Roets F, Crous PW, Dreyer LL. 2005. Seasonal trends in colonization of *Protea* infructescences by *Gondwanamyces* and *Ophiostoma* spp. S Afr J Bot 71:307–311.

———, de Beer ZW, Dreyer LL, Zipfel R, Crous PW, Wingfield MJ. 2006. Multigene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. Stud Mycol 55:199–212.

———, Crous PW, Wingfield MJ, Dreyer LL. 2007. Discovery of fungus-mite-mutualism within a unique niche of the Cape Floral Kingdom. Environ Entomol 36 (In press).

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Saitou N, Nei M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Shimodaira H, Hasegawa M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol Biol Evol 16:1114–1116.

Sinclair WA, Lyon HH. 2005. Diseases of trees and shrubs. 2nd ed. New York: Cornell University Press.

Six DL, Paine TD. 1998. Effects of mycangial fungi on host tree species progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). Environ Entomol 27:1393–1401.

Stephens RB. 1974. Mycology guidebook. Seattle: University of Washington.

Swofford DL. 2000. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0bla. Massachusetts: Sinauer Associates.

Tavare S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Lect Mat Life Sci 17: 57–86.

Upadhyay HP. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. Athens: University of Georgia Press.

White TJ, Bruns TD, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. New York: New York Academic Press. p 315–322.

Whitney HS. 1982. Relationships between bark beetles and symbiotic organisms. In: Mitton JB, Sturgeon KB, eds. Bark beetles in North American conifers. Austin: University of Texas Press.

Wingfield MJ, Seifert KA, Webber JF. 1993. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. St Paul, Minnesota: APS Press.

Zhou XD, de Beer ZW, Ahumada R, Wingfield BD, Wingfield MJ. 2004. *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. Fungal Divers 15:261–274.

———, ———, Wingfield MJ. 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with bark beetles in South Africa. Stud Mycol 55:269–277.

Zipfel RD, de Beer ZW, Jacobs K, Wingfield BD, Wingfield MJ. 2006. Multigene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Stud Mycol 55:75–97.